UNIVERSITI PUTRA MALAYSIA

HYOSCYAMINE AND SCOPOLAMINE PRODUCTION IN TRANSFORMED ROOT CULTURES OF *DATURA METEL* L

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HYOSCYAMINE AND SCOPOLAMINE PRODUCTION IN TRANSFORMED ROOT CULTURES OF *DATURA METEL* L.

By

AZIZ BIN AHMAD

Thesis Submitted in Fulfilment of the Requirement for the Degree of Doctor of Philosophy in the Faculty of Science and Environmental Studies
Universiti Putra Malaysia

December 2000

Dedicated to my Family…….

Abstract of the thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

HYOSCYAMINE AND SCOPOLAMINE PRODUCTION IN TRANSFORMED ROOT CULTURES OF *DATURA METAL* L.

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AZIZ BIN AHMAD

December 2000

Chairperson: Prof. Dr. Marziah Mahmood

Faculty: Science and Environmental Studies

The transformed root cultures of *Datura metel* L (kecubung) was successfully established *via Agrobacterium rhizogenes*-mediated, which contained the pBI 121 plasmid harbouring the GUS and kanamycin coding genes. The transformation was biochemically confirmed with GUS assay indicated by the presence of blue spot on roots, Southern blotting and the resistance of transformed root to Kanamycin. Transformed roots were showed a typical character of transformed root, which is sensitive to exogenous IAA (auxin). Sustained root cultures appeared to produce hyoscyamine and scopolamine ten times higher than that produced in the intact plant.

The ability of the transformed roots to produce hyoscyamine and scopolamine in different types of basal media used was examined. It was observed that Gamborg's B5 basal medium was the best medium for root growth as well as the hyoscyamine and scopolamine production. Gamborg's B5 medium was used for subsequent studies. Among the carbon source tested, sucrose appeared to be the best carbon source for root growth. Consequently, the effect of Gamborg's B5 medium ionic strength and sucrose concentration was examined. Gamborg's B5 medium was

used in quarter, half and full strength, and supplemented with sucrose concentration in the range of 1 – to 8 % (w/v). Full strength Gamborg's B5 medium with 4 % sucrose was observed to enhance the root growth as well as hyoscyamine and scopolamine production. Studies were also carried out to examine the effect of various concentrations of macro and microelements on root growth, hyoscyamine and scopolamine production. The macro elements used were nitrogen (ammonium and nitrate balance), magnesium, calcium and phosphate. Amongst the macro element tested, nitrogen, which is in the form of nitrate and/or ammonium, was found to have a significant effect on the hyoscyamine and scopolamine production. Meanwhile, the microelements that have been studied were copper, ferric, manganese, zinc and boron. Ferric and copper appeared to have the greater effect on hyoscyamine and scopolamine production than other elements. Roots cultured in medium with lower concentration of microelement than that present in Gamborg's B5 medium was also observed to enhance the hyoscyamine and scopolamine content.

Feeding of each precursors i.e. putrescine, l-ornithine, arginine, l-phenylalanine, hyoscyamine and scopolamine at lower concentration (less than 0.2 mM) into treatment medium was observed to reduce the root growth as well as the hyoscyamine and scopolamine production. Combination of *l*-phenylalanine with putrescine appeared the best precursor for both hyoscyamine and scopolamine production.

Abstrak thesis dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENGHASILAN HIOSIAMINA DAN SKOPOLAMINA DALAM KULTURA AKAR *DATURA METEL* L. YANG TELAH DITRANSFORMASI

Oleh

AZIZ BIN AHMAD

Disember 2000

Pengerusi: Prof. Dr. Marziah Mahmood

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Kultura akar rerambut kecubung (*Datura metel*) telah berjaya dihasilkan melalui perantaraan *Agrobacterium rhizogenes*, yang mengandungi plasmid pBI 121 yang membawa kod genetik bagi GUS dan Kanamycin. Tramsformasi telah disahkan dengan pembentukan warna biru pada akar oleh ujian GUS, Southern blot dan ketahanan akar ditransformasi pada Kanamycin. Akar ditransformasi juga menunjukkan ciri istimewa iaitu peka terhadap IAA luaran. Kultur akar yang dihasilkan juga menghasilkan hiosiamina dan skopolamina sepuluh kali lebih tinggi daripada yang dihasilkan oleh induknya.

Keupayaan akar untuk menghasilkan hiosiamina dan skopolamina dalam media asas yang berbeza telah diuji. Media Gamborg's B5 telah dikenalpasti sebagai media yang terbaik untuk pertumbuhan akar, begitu juga penghasilan hiosiamina dan skopolamina. Justeru itu, media Gamborg's B5 telah dipilih untuk kajian selanjutnya. Diantara gula yang diuji, sukrosa merupakan sumber karbon terbaik untuk pertumbuhan akar. Sebagai urutan, kesan kepekatan ionik media Gamborg's B5 dan sukrosa telah diuji. Media Gamborg's B5 telah digunakan pada kepekatan

suku, separuh dan penuh dan dibekalkan degan sukrosa pada julat 1 - ke 8 % (b/i). Kepekatan penuh media Gamborg's B5 dengan 4 % sukrosa didapati telah mengggalakkan pertumbuhan akar, begitu juga penghasilan hiosiamina dan skopolamina. Kajian juga telah dijalankan terhadap kesan pelbagai kepekatan unsur makro dan mikro terhadap pertumbuhan akar, penghasilan hiosiamina dan skopolamina. Unsur makro yang telah digunakan ialah nitrogen (ammonia dan nitrat), magnesium, kalsium dan fosfat. Di antara unsur makro yang telah diuji, nitrogen dalam bentuk nitrat dan/atau ammonia didapati boleh menunjukkan kesan yang signifikan terhadap penghasilan hiosiamina dan skopolamina. Sementara itu, unsur mikro yang telah diuji adalah kuprum, ferik, mangan, zink dan boron. Ferik dan kuprum menunjukkan kesan yang besar terhadap penghasilan hiosiamina dan skopolamina. Kultura akar dalam media dengan kepekatan unsur mikro yang lebih rendah daripada unsur di dalam medium Gamborg's B5 didapati merangsang penghasilan hiosiamina dan skopolamina.

Rawatan penambahan setiap prekursor berikut; putrescina, l-ornithin, arginina, l-fenilalanina, hiosiamina dan skopolamina.ke dalam medium pada kepekatan yang lebih rendah (kurang daripada 0.2 mM) didapati memberi kesan terhadap pertumbuhan akar dan penghasilan hiosiamina serta skopolamina. Kombinasi l-fenilalanina dengan putrescine merupakan prekursor terbaik bagi penghasilan hiosiamina dan skopolamina.

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Last but not least, my heartiest appreciation to my parent, my brothers and sisters for giving the support and strength during my studies.

!! May **ALLAH** bless us always !!

I certify than an Examination Committee met on 20th December, 2000 to conduct the final examination of Aziz bin Ahmad on his Doctor of Philosophy thesis entitled "Hyoscyamine and Scopolamine Production in Transformed Root Cultures of *Datura metel* L." in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulation 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows.

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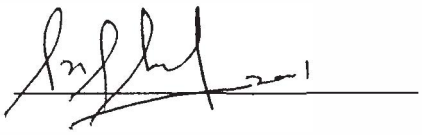
DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other degree at UPM or other institutions.



(AZIZ BIN AHMAD)

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURE

LIST OF PLATES

LIST OF ABBREVIATIONS

BAP	benzyl-amino purine
DNA	deoxyribonucleic acid
DMRT	Duncan's Multiple Ranges Test
g	grams
GA_3	gibberellic acid
GUS	β-Glucuronidase
HPLC	High Performance Liquid Chromatography
IAA	indole-acetic acid
mg	milligrams
mg/g	milligrams per gram
mg/L	milligrams per litre
mL	millilitre
mM	millimolar
N	normal
µg	micrograms
µg/g	micrograms per gram
µM	micro molar
wt.	weight
w/v	weight per volume
v/v	volume per volume
^0C	degree of centigrade
%	percentage

CHAPTER I

INTRODUCTION

Use of Hyoscyamine and Scopolamine

Tropane alkaloids contain more than 150 members including hyoscyamine and scopolamine (Yamada and Tabata, 1997). They were found in many genera of Solanaceae family, e.g. *Atropa, Brugmansia, Datura, Duboisia, Hyoscyamus, Mandragora*, and *Scopolia* (Yamada and Tabata, 1997). *Datura* species are plants that produce the largest amount of hyoscyamine and scopolamine (Jung and Tepfer, 1987). Atropine (racemate of *d* and *l*-hyoscyamine) and scopolamine are anti-choligernic agent or as parasympatholytic agents (Jauhikainen et al., 1999; Pitta-Alvarez and Giulietti, 1995). *l*-hyoscyamine first stimulates, then depresses the central nerve system (CNS), whereas *l*-scopolamine depresses it, because of this property scopolamine is preferred in clinical application and the demand in the world-market is 10 times more than that of *l*-hyoscyamine (Yamada and Tabata, 1997). A combination of scopolamine and hyoscyamine was used with ergotamine for treating acute migraine (Lewis and Elvin-Lewis, 1977). Scopolamine itself was used for treating motion sickness, sleeping pill psychosis and as anti-inflammatory on eyes (Pitta-Alvarez and Giulietti, 1995). Meanwhile, hyoscyamine was used for treating asthma, ulcer, Parkinson's disease and also used as anti spasmodic and analgesic (Lewis and Elvin-Lewis, 1977). However, the production of these particular compounds was extracted directly from any part of the plant. This accounted with the yield and the productivity was not constant due to uncertainty of supply and instability of raw materials (Yeoman and Yeoman, 1996). Therefore, to overcome this problem artificial chemical synthesis was introduced.

However, the biochemicals have complex structures and can be chiral molecules, which are difficult and expensive or even impossible to synthesize chemically (Waterman, 1992; Leete 1990). Subsequently, according to Hibi et al., (1992) only *l*-isomer of these tropane alkaloids is pharmacologically active.

Plant Tissue Culture

Plant tissue culture offers an alternative approach for the production and manufacturing of natural and foreign plant secondary products. Plant cell culture, however, was reported oftenly fail to produce the spectrum of the useful compounds in the higher plants and the yield was low which became a major barrier for their production (Yeoman and Yeoman, 1996; Oksman-Caldentey et al. 1994; Constabel, 1990). Development of plant organ cultures such as roots, shoots and leaves was shown to enhance the production of secondary metabolites *in vitro* (Subroto et al., 1996a,b; Ehmke et al., 1995; Alvarez et al., 1994; Sharp and Doran, 1990). In recent years, there has been an increasing interest in application of genetically transformed organs such as transformed root cultures for plant secondary metabolite production.

Transformed roots could be obtained following the infection of explant with *Agrobacterium rhizogenes*. This was due to the transfer and cooperation of Ri T-DNA from the bacteria into genomes of the host plant cells (Chilton et al., 1992). This T-DNA was indicated to alter auxin metabolism in plant cells (Hamill, 1993) and resulted in a rapid growth of roots called `hairy roots'. This kind of culture was reported to produce higher secondary metabolite than normal plant with a similar spectrum (Jung and Tepfer, 1987). The ability of transformed root cultures to produce tropane alkaloids has been demonstrated for several genera in Solanaceae.

These include *Datura*, *Atropa*, *Scopolia*, *Hyoscyamus*, *Brugmansia* and *Duboisia* (Maldonado-Mendoza et al., 1995; 1993; Robins et al., 1990; Rhodes et al., 1989; Jaziri et al., 1988). These alkaloids are synthesised in the roots then transported to and accumulated in the leaves (Mano et al., 1989; Endo and Yamada, 1985). Therefore, using the plant root cultures is the best system for the producing these particular alkaloids.

Objectives

1. To establish the transformed hairy root culture of *Datura metel*.
2. To determine the effect of plant growth regulators and media manipulation on root growth, scopolamine and hyoscyamine production.
3. To investigate the influence of different macro and microelements and precursors on root growth, scopolamine and hyoscyamine production in hairy root cultures.

CHAPTER II

LITERATURE REVIEW

Root Properties

The main function of plant organ such as roots is as water and nutrient absorber (Canny, 1998). However, roots have been demonstrated to play an important role for biosynthesis of plant secondary metabolites. Studies have shown that roots was the main organ used for biosynthesis of terpenoids, steroids, alkaloids and phenolics in the higher plants (Harbone and Khan, 1993; Parr, 1989; Endo and Yamada, 1985). Hashimoto et al., (1991), reported that root is the main site for biosynthesis of tropane alkaloids. These particular alkaloids were then naturally transported through a vascular system-xylem and phloem (Kitamura et al., 1993) and accumulated in the leaves or other parts of plant (Mano et al., 1989; Ghani, 1986; Endo et al., 1987). The production of hyoscyamine is fulfilled in the roots of the plant, while its transformation (epoxide) to scopolamine is accomplished either in pericycle in some plants (Kanagae et al., 1994) or in the aerial parts in others (Maldonado-Mendoza and Loyola-Vargas, 1995; Yun et al., 1992; Parr, 1989).

Root Culture

The basic techniques required in initiating and maintaining excised root cultures were established as early as the 1930s. Butcher and Street (1964) reviewed further modification and refinements in the methodology (Charlwood et al., 1990). Auxins at lower concentrations were normally used for initiating and growth of the roots